

# Tight control of gene expression in mammalian cells by tetracycline-responsive promoters

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**ABSTRACT** Control elements of the tetracycline-resistance operon encoded in *Tn10* of *Escherichia coli* have been utilized to establish a highly efficient regulatory system in mammalian cells. By fusing the *tet* repressor with the activating domain of virion protein 16 of herpes simplex virus, a tetracycline-controlled transactivator (tTA) was generated that is constitutively expressed in HeLa cells. This transactivator stimulates transcription from a minimal promoter sequence derived from the human cytomegalovirus promoter IE combined with *tet* operator sequences. Upon integration of a luciferase gene controlled by a tTA-dependent promoter into a tTA-producing HeLa cell line, high levels of luciferase expression were monitored. These activities are sensitive to tetracycline. Depending on the concentration of the antibiotic in the culture medium (0–1 µg/ml), the luciferase activity can be regulated over up to five orders of magnitude. Thus, the system not only allows differential control of the activity of an individual gene in mammalian cells but also is suitable for creation of “on/off” situations for such genes in a reversible way.

The study of gene function in complex genetic environments such as mammalian cells would greatly profit from systems that would allow stringent control of the expression of individual genes. Ideally, such systems would not only mediate an “on/off” situation of gene activity but also would permit limited expression at a defined level.

Attempts to control gene activity by various inducible eukaryotic promoters responsive to, for example, heavy metal ions (1–3), heat shock (4), or hormones (5–8) have generally suffered from leakiness of the inactive state (e.g., the metallothionein promoter; ref. 1) or from pleiotropic effects caused by the inducing principles themselves, such as elevated temperature or glucocorticoid hormone action (9).

In search of regulatory systems that do not rely on endogenous control elements, several groups, including ours, have demonstrated that the *lac* repressor/operator/inducer system of *Escherichia coli* functions in mammalian cells. Three basically different approaches have been described: (i) prevention of transcription initiation by properly placed *lac* operators at promoter sites (10–14), (ii) blockage of transcribing RNA polymerase II during elongation by a *lac* repressor/operator complex (*lacR/O*; ref. 15), and (iii) activation of a promoter responsive to a fusion between *lacR* and the activating domain of virion protein 16 (VP16) of herpes simplex virus (HSV) (16, 17).

At present, however, the utility of the *lacR/O*-based systems in mammalian cells is limited since the inducer isopropyl β-D-thiogalactopyranoside (IPTG), despite its rapid uptake and intracellular stability (18), acts rather slowly and inefficiently, resulting in only moderate induction. Nevertheless, an interesting conditional mutant of a *lacR*-VP16 fusion has been described (17). It activates a minimal promoter ≈1000-fold at elevated temperatures in the presence of

IPTG. The temperature dependence and the inherent IPTG-related problems, however, may once again limit this approach.

Here we describe a control system that in HeLa cells allows regulation of expression of an individual gene over up to five orders of magnitude. This system is based on regulatory elements of the *Tn10*-specified tetracycline-resistance operon of *E. coli* (19), in which transcription of resistance-mediating genes is negatively regulated by the tetracycline repressor (*tetR*). In the presence of the antibiotic tetracycline *tetR* does not bind to its operators located within the promoter region of the operon and allows transcription. By combining *tetR* with the C-terminal domain of VP16 from HSV, known to be essential for the transcription of the immediate early viral genes (20), a hybrid transactivator was generated that stimulates minimal promoters fused to tetracycline operator (*tetO*) sequences. These promoters are virtually silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to *tetO* sequences.

The specificity of the *tetR* for its operator sequence (19) as well as the high affinity of tetracycline for *tetR* (21) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an inducible expression system in mammalian cells far superior to the *lacR/O/IPTG* system. This has already been demonstrated in plant cells, in which direct repressor action at promoter sites is efficiently reversed by the antibiotic (22, 23).

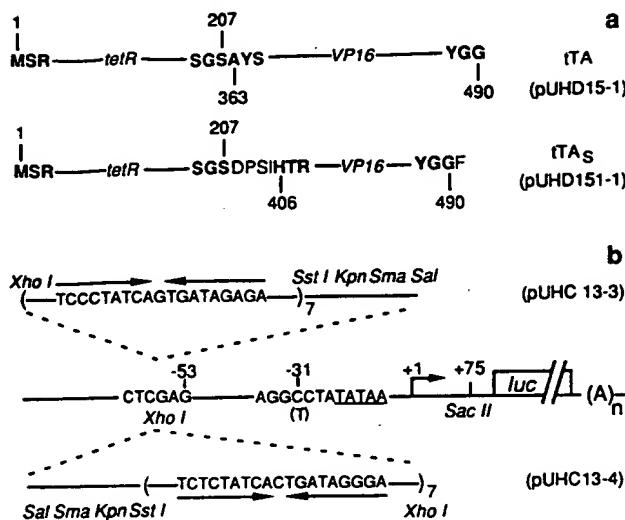
## MATERIALS AND METHODS

**Construction of the Transactivators tTA and tTA<sub>s</sub>.** The *tetR* sequence was originally recovered from pWH510 (24) by PCR and inserted into pUHD10-1 (14), resulting in pUHD14-1 (A. Bonin and H.B., unpublished). A unique *Afl* II cleavage site overlapping the *tetR* stop codon in this plasmid construct allows for the in-frame insertion of coding sequences. To generate tTA, a 397-base-pair (bp) *Mlu* I/*Fok* I fragment of pMSVP16 (20) coding for the C-terminal 130 amino acids of VP16 of HSV was blunted by filling in the protruding ends with T4 DNA polymerase. This DNA was inserted into pUHD14-1 previously cleaved with *Afl* II and blunted by mung bean nuclease. The resulting plasmid pUHD15-1 encodes the tTA sequence (Fig. 1a) under the control of the *P<sub>hCMV</sub>* (human cytomegalovirus promoter IE; see below). In a homologous approach a DNA fragment coding for the 97-amino acid C-terminal portion of VP16 was fused to *tetR* by PCR-mediated cloning. The resulting plasmid, pUHD151-1, encodes the smaller version of the transactivator, tTA<sub>s</sub> (Fig. 1a).

**Abbreviations:** tTA, tetracycline-controlled transactivator; IPTG, isopropyl β-D-thiogalactopyranoside; HSV, herpes simplex virus; hCMV, human cytomegalovirus; VP16, virion protein 16; tk, thymidine kinase; P, promoter; rlu, relative light units; lacR/O, lac repressor/operator complex; tetR, tetracycline repressor; tetO, tetracycline operator; SV40, simian virus 40.

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**FIG. 1.** Schematic representation of the *tetR*-VP16 fusion proteins and the tTA-dependent transcription unit. (*a*) Outline of the two tTA proteins. In both fusion proteins, tTA and tTA<sub>s</sub>, the original 207-amino acid sequence of *tetR* is conserved. Two versions of VP16 sequences encoding the activation domain were fused in frame to the 3' end of the *tetR* gene, resulting in tTA and tTA<sub>s</sub>. The bold letters indicate the original amino acids at the N terminal end, the junction, and the C-terminal end of the fusion proteins; the other letters designate amino acids introduced due to sequence constraints. The numbers delineate amino acid positions within *tetR* (19) or VP16 (20), respectively. (*b*) The tTA-dependent transcriptional unit consists of the simian virus 40 (SV40) poly(A) site (A<sub>n</sub>), the luciferase gene (luc), and P<sub>CMV\*-1</sub> or P<sub>hCMV\*-2</sub>. The two promoters encompass the sequence between +75 and -53 of the P<sub>hCMV</sub>, with one base-pair exchange at -31, which creates a *Sst* I cleavage site. The *Xho* I site introduced at -53 by PCR was utilized to insert the heptamerized *terO* sequence. This heptamer sequence is flanked at one side by an 18-nucleotide polylinker, which allows the insertion of the operators in both orientations as *Sal* I/*Xho* I fragments. The position of the central G/C base pair of the promoter proximal operator to position +1 is -95 for P<sub>CMV\*-1</sub> (upper construct) and -76 for P<sub>CMV\*-2</sub> (lower construct). The plasmids that contain the four constructs are indicated on the far right.

**Construction of P<sub>hCMV\*</sub> and the Luciferase Reporter Plasmid.** Plasmid pUHC13-1 is a derivative of pUHD10-1 (14) and was originally developed by U. Deuschele in our laboratory. It contains the promoter-enhancer sequence of P<sub>hCMV</sub>, spanning position +75 to position -675 (25). This promoter is followed by a polylinker and the luciferase gene of *Photinus pyralis* fused to the SV40 small-t intron and poly(A) signal. The latter elements and the luciferase gene were transferred from pSV2L-ΔΔ5' (26). By this transfer the N terminus of luciferase has been modified as described (14). The enhancer region of P<sub>hCMV</sub> was removed by PCR-mediated cloning, whereby a *Xho* I site was introduced adjacent to position -53. The resulting minimal promoter, P<sub>hCMV\*</sub> (Fig. 1*b*) is part of the reporter plasmid pUHC13-2.

**Construction of P<sub>hCMV\*-1</sub> and P<sub>hCMV\*-2</sub>.** To combine P<sub>hCMV\*</sub> with *ter* operators, the 19-bp inverted repeat sequence of operator O2 of Tn10 (19) was synthesized as part of a 42-bp DNA fragment (upper strand: 5'-TCGAGTTTAC-CACTCCCTATCAGTGATAGAGAAAAGTGAAG-3'). Upon annealing, the two complementary strands exposed the compatible protruding ends of a *Xho* I and a *Sal* I cleavage site at the 5' and 3' ends, respectively. Ligation of this fragment into the *Xho* I site of the polylinker of pT81-luc (27) created upon cloning single as well as multiple inserts of operator sequences upstream of a thymidine kinase (tk) minimal promoter from HSV contained in pT81-luc. tk promoters containing one, two, and seven operator sequences were examined for their ability to be activated in transient

expression experiments using the HeLa cell line HtTA-1 (see below). All constructs were active in tTA-producing cells in a tetracycline-dependent manner. The heptameric version of the *terO* sequence is caused by far the highest activation of all P<sub>tk</sub>-*terO* constructs. It therefore was removed as a *Xho* I/Sal I fragment and transferred into pUHC13-2. Due to the asymmetric location of the *terO* within the polylinker of pT81-luc, the resulting plasmids pUHC13-3 and pUHC13-4 contain the heptameric *terOs* in two orientations differing in the distance between the operators and position +1 of P<sub>hCMV</sub> by 19 bp. The two *terO*-containing promoters were designated P<sub>hCMV\*-1</sub> and P<sub>hCMV\*-2</sub> (Fig. 1*b*).

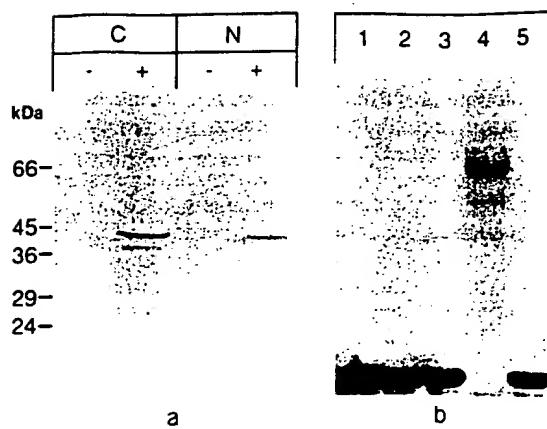
**Band-Shift Assay.** Cytoplasmic and nuclear cell extracts from  $\approx 2 \times 10^6$  cells were prepared as described by Andrews and Faller (28) except that the cytoplasmic protein fraction was centrifuged once more (1 hr, 100,000  $\times g$ ). Nuclear proteins were extracted by a buffer containing 20 mM Hepes-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Aliquots (5  $\mu$ l) of nuclear extracts were mixed with 15  $\mu$ l of binding buffer (10 mM Tris-HCl, pH 7.5/10 mM MgCl<sub>2</sub>) containing 20  $\mu$ g of calf thymus DNA, 5  $\mu$ g of bovine serum albumin, and 2 fmol of  $^{32}$ P-labeled *terO* DNA. The *terO* DNA was isolated from pUHC13-3 as a 42-bp *Taq* I fragment whose protruding ends were filled in by Klenow enzyme in the presence of [ $\alpha$ - $^{32}$ P]dCTP. After 20 min at room temperature, aliquots of the binding reaction mixture were loaded onto a 5% polyacrylamide/0.07% bisacrylamide gel. Electrophoresis was carried out in 90 mM Tris base/90 mM boric acid/3 mM EDTA at 5 V/cm.

**Luciferase Assays.** Cells grown to  $\approx 80\%$  confluence in 35-mm dishes in Eagle's minimum essential medium were washed with 2 ml of phosphate-buffered saline before they were lysed in 25 mM Tris phosphate, pH 7.8/2 mM dithiothreitol/2 mM diaminocyclohexanetetraacetic acid/10% glycerol/1% Triton X-100 for 10 min at room temperature. The lysate was scraped off the culture dishes and centrifuged for 10 sec in an Eppendorf centrifuge. Next, aliquots (10  $\mu$ l) of the supernatant were mixed with 250  $\mu$ l of 25 mM glycylglycine/15 mM MgSO<sub>4</sub>/5 mM ATP and assayed for luciferase activity in a Lumat LB9501 (Berthold, Wildbad, F.R.G.) using the integral mode (10 sec). D-Luciferin (L6882, Sigma) was used at 0.5 mM. The background signal measured in extracts of HeLa cells that did not contain a luciferase gene was indistinguishable from the instrumental background [80–120 relative light units (rlu)/10 sec]. Protein content of the lysates was determined according to Bradford (29).

## RESULTS

**Construction and Characterization of the tTA.** To convert the prokaryotic *tet* repressor into a eukaryotic transactivator it was fused to the negatively charged C-terminal domain of HSV-VP16, known to be essential for transactivation (20). Sequences coding for either a 97- or a 127-amino acid C-terminal portion of VP16 were fused to the *tetR* gene, resulting in the coding sequences of tTA<sub>s</sub> and tTA, respectively (Fig. 1*a*). In plasmids coding for tTA (pUHD15-1) or tTA<sub>s</sub> (pUHD151-1) the transactivator sequences are flanked upstream by P<sub>hCMV</sub> and downstream by the SV40 poly(A) site. Since both fusion proteins did not differ in their functional *in vivo* properties, only the results obtained with the larger version, tTA, are reported here.

HeLa cells transiently transfected with pUHD15-1 produced a fusion protein of the expected molecular mass (37 kDa), as demonstrated in immunoblots of the electrophoretically separated cytoplasmic and nuclear extracts (Fig. 2*a*). When nuclear extracts were mixed with the *terO* DNA the electrophoretic mobility of the DNA was diminished. The specificity of the interaction between tTA and operator DNA



**FIG. 2.** Identification and characterization of tTA produced in HeLa cells. HeLa cells grown to 40% confluence were transiently transfected with pUHD15-1 by the calcium phosphate method. Nuclear and cytoplasmic extracts were prepared after 36 hr. (a) Western blot analysis of electrophoretically separated extracts (6% acrylamide/0.1% SDS gels) with *tetR*-specific antibodies reveals a protein of about 37 kDa (tTA) in cytoplasmic (C) and nuclear (N) extracts in pUHD15-1 transfected cells (+) that is not present in mock-transfected cells (-). (b) Mobility change of *tetO* DNA by tTA binding from HeLa cell nuclear extracts. Radioactively labeled *tetO* DNA was mixed with extracts from mock-transfected (lanes 2 and 3) and pUHD15-1-transfected (lanes 4 and 5) HeLa cells in the absence (lanes 2 and 4) and presence (lanes 3 and 5) of 1  $\mu$ g of tetracycline per ml (added 2 min prior to the addition of the operator). Lane 1 contains labeled operator DNA only.

was confirmed by the finding that no mobility change for *tetO* DNA was detectable in the presence of the specific inducer tetracycline (Fig. 2b).

**Construction of a tTA-Dependent Promoter.** To generate promoters activatable by tTA, *tetO*s were inserted upstream of minimal promoter sequences. For *P<sub>hCMV</sub>* the upstream enhancer region was removed by PCR and a *Xho* I cleavage site was introduced adjacent to position -53. This minimal promoter, designated *P<sub>hCMV\*</sub>*, spans the original *P<sub>hCMV</sub>* sequence from +75 to -53 (+1 being the first nucleotide transcribed) and, in addition, contains a *Stu* I site around -31 (Fig. 1b). *tetO* sequences were fused to this core promoter by insertions at the *Xho* I site (Fig. 1).

The *tetO* sequence O2 of *Tn10* is a 19-bp inverted repeat to which *tetR* binds as a 46-kDa dimer (19). It was chemically synthesized and ligated into the *Xho* I cleavage site of the polylinker located upstream of the minimal tk promoter in plasmid pT81-luc (27). Multiple insertions of *tetO*s created a set of promoters that contained between 1 and 7 *tetO* sequences upstream from position -81 of the tk promoter. A *Xho* I/*Sal* I fragment containing 7 *tetO*s fused head to tail was recovered from one of the constructs and transferred into the *Xho* I site upstream of *P<sub>hCMV\*</sub>*. Due to the asymmetry of the *Xho* I/*Sal* I fragment, two *P<sub>hCMV\*</sub>-tetO* constructs were obtained that differ in the distance between the operators and position +1 of *P<sub>hCMV\*</sub>*, which is 95 bp for *P<sub>hCMV\*-1</sub>* and 76 bp for *P<sub>hCMV\*-2</sub>*. The plasmids containing these promoters are designated pUHC13-3 and pUHC13-4, respectively (Fig. 1b). When HeLa cells were transiently transfected with these plasmids high levels of luciferase activity were monitored whenever the cells were cotransfected with pUHD15-1, which provided the coding sequence of tTA. Little activity was observed with cultures grown in the presence of tetracycline (1.0  $\mu$ g/ml) or with plasmids containing *P<sub>hCMV\*</sub>* only (data not shown). Since *P<sub>hCMV\*-1</sub>* and *P<sub>hCMV\*-2</sub>* were activated by tTA to a significantly higher degree than any of the *P<sub>tk</sub>* constructs, the latter ones were not investigated further.

**Quantitation of *P<sub>hCMV\*-1</sub>* and *P<sub>hCMV\*-2</sub>* Activation by tTA.** To quantify the stimulation of *P<sub>hCMV\*-1</sub>-tetO* constructs by tTA, HeLa cell lines were established that contained the *P<sub>hCMV\*-1</sub>* or the *P<sub>hCMV\*-2</sub>-luciferase* as well as the *P<sub>hCMV</sub>-tTA* expression units stably integrated. Conditions for cultivating and selecting cells have been described (14). In a first step cells were cotransfected with pUHD15-1 and pSV2n $\alpha$  (30). Clones resistant to G418 were assayed for transactivation of *P<sub>hCMV\*-1</sub>* by transient transfection with pUHC13-3. In all HeLa cell clones in which the tetracycline-responsive promoters were active, tTA was not detectable by Western blots or by immunofluorescence. Its presence was just barely visible in electrophoretic mobility shift experiments of highly labeled *tetO* DNA. This indicates very low intracellular concentrations of tTA and may reflect a selection against squelching effects caused by higher concentrations of VP16-activating domains (31).

One of the positive clones, HtTA-1, was then cotransfected with a plasmid carrying the hygromycin-resistance gene (pHMR272; ref. 32) and either pUHC13-3 or pUHC13-4, resulting in the X and T series of clones, respectively. Clones resistant to hygromycin and G418 were assayed for luciferase activity. As shown in Table 1, in the absence of tetracycline this activity differed in individual clones by almost four orders of magnitude. However, in all cases the luciferase activity was sensitive to tetracycline in the culture. This demonstrates that the expression of luciferase is dependent on the function of tTA, which obviously is capable of activating promoter constructs *P<sub>hCMV\*-1</sub>* and *P<sub>hCMV\*-2</sub>*.

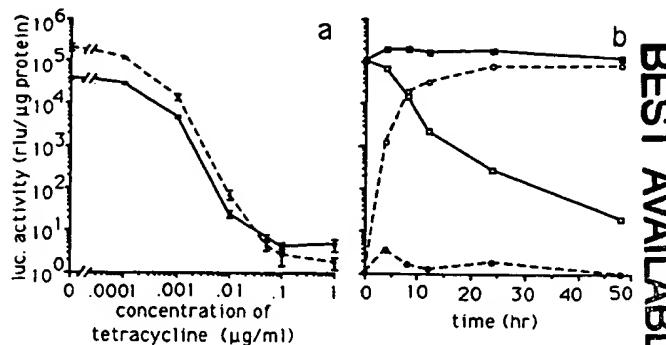
When the luciferase activity within various clones was monitored in the presence and absence of tetracycline hydrochloride (Sigma), two remarkable results emerged. (i) In all clones tested, tTA greatly stimulated promoter activity, even up to five orders of magnitude in clone X1. (ii) In clones T14, T16, X1, and X2 (Table 1) tetracycline reduced luciferase activity to values that cannot be quantified even at high protein concentrations of the extracts due to instrumental limitations (i.e., rlu/ $\mu$ g of protein  $\leq$  2). This demonstrates that *P<sub>hCMV\*-1</sub>* and *P<sub>hCMV\*-2</sub>* are virtually silent when integrated in the proper genomic environment and that their activity depends exclusively on the action of tTA.

The tTA inactivation studies were carried out with 1  $\mu$ g of tetracycline per ml in the culture medium. A partial inactivation of tTA is, however, readily achieved with tetracycline concentrations below 0.1  $\mu$ g/ml, as shown in Fig. 3a. In the

**Table 1.** Tetracycline-dependent luciferase activity of different HeLa cell clones

Clone	Luciferase activity, rlu/ $\mu$ g of protein		Activation factor
	With Tc	Without Tc	
T7	1074 $\pm$ 75	79,197 $\pm$ 2,119	7.3 $\times$ 10 <sup>1</sup>
T11	2.5 $\pm$ 0.4	34,695 $\pm$ 1,127	1.3 $\times$ 10 <sup>4</sup>
T12	3.5 $\pm$ 0.9	35,298 $\pm$ 5,009	1 $\times$ 10 <sup>4</sup>
T14	$\leq$ 2	33 $\pm$ 4	$\geq$ 1.5 $\times$ 10 <sup>1</sup>
T15	286 $\pm$ 47	49,070 $\pm$ 2,784	1.7 $\times$ 10 <sup>2</sup>
T16	$\leq$ 2	541 $\pm$ 133	$\geq$ 2.7 $\times$ 10 <sup>2</sup>
X1	$\leq$ 2	257,081 $\pm$ 40,137	$\geq$ 1 $\times$ 10 <sup>5</sup>
X2	$\leq$ 2	104,840 $\pm$ 20,833	$\geq$ 5 $\times$ 10 <sup>4</sup>
X7	75 $\pm$ 7	125,745 $\pm$ 18,204	1.6 $\times$ 10 <sup>3</sup>

The HeLa cell clone HtTA-1, which constitutively expresses tTA, was cotransfected with pUHC13-3 or pUHC13-4 and pHMR272. Hygromycin-resistant clones were examined for luciferase activity. Nine clones identified were subcloned and luciferase activity was quantified in the presence (1  $\mu$ g/ml) and absence of tetracycline (Tc). Values are arithmetic means of three independent luciferase determinations (from three independently grown cultures). Luciferase activities of <2 rlu/ $\mu$ g of protein are too close to the instrumental background to be quantified.



**FIG. 3.** Dependence of tTA function on tetracycline. (a) Dependence of luciferase (luc.) activity on tetracycline concentration. HeLa cell clones X1 (dashed line) and T12 previously grown in tetracycline-free medium were seeded with a density of 5000 cells per 35-mm dish and incubated at the tetracycline concentrations indicated. After reaching ≈90% confluence, cells were harvested and assayed for luciferase activity. Data given are the means ± SD of three independent experiments. (b) Kinetics of tetracycline action. X1 cells were grown in 100-mm dishes to ≈80% confluence in the absence or presence (0.1 μg/ml) of tetracycline. At time 0 cells were washed with phosphate-buffered saline and split into smaller culture dishes (1/20th of the initial cultures per 35-mm dish). Half of the cultures remained in tetracycline-free medium (■) and the other half was incubated in the presence of tetracycline (1 μg/ml; □). The X1 culture grown in tetracycline-containing medium was split in the same manner: one half was further incubated in the presence of tetracycline (●), whereas the other half was transferred to tetracycline-free medium (○). At the times indicated, aliquots were harvested and examined for luciferase activity. The slight increase in luciferase activity monitored at 4 hr in the culture containing tetracycline (●) is reproducible and reflects luciferase induction during the washing step.

two clones analyzed (T12 and X1), a stepwise reduction of the tetracycline concentration in the medium gradually increased the luciferase activity. These results again demonstrate that, in the case of clone X1, tTA can regulate transcriptional activity, as monitored by luciferase activity, by over five orders of magnitude. Moreover, we find that at tetracycline concentrations sufficient for full inactivation of tTA (0.1 μg/ml), no change in growth behavior or morphology of HeLa cells occurs. Only at tetracycline concentrations well above 10 μg/ml were such changes observed upon prolonged incubation (data not shown).

**Kinetics of Tetracycline Action.** The time course of tetracycline action was analyzed in cultures grown in the absence or presence of tetracycline. At time 0 the antibiotic was added to the tetracycline-free cultures (final concentration, 1 μg/ml), whereas the tetracycline-containing cultures were rinsed and incubated in fresh, antibiotic-free medium (Fig. 3b). At various times cells were harvested and analyzed for luciferase activity. As shown in Fig. 3b, the depletion of tetracycline leads to a rapid induction of luciferase activity, reaching >20% of the fully induced level within 12 hr. A similarly rapid reduction of luciferase activity was observed when tetracycline was added to the fully active tetracycline-free system: within 8 hr, activity dropped to about 10% and reached <2% of its original value after 12 hr.

## DISCUSSION

The fusion of the *Tn10*-derived *E. coli* *tetR* with the activation domain of VP16 from HSV has generated a transactivator exhibiting all of the properties required for the specific and stringent regulation of an individual gene in a mammalian cell. The transactivator tTA produced in HeLa cells binds specifically to *tetO* sequences *in vitro*. This association is prevented by tetracycline. When bound to *tetOs* placed

upstream of minimal promoters, tTA efficiently activates transcription from such promoters *in vivo* in a tetracycline-dependent manner. The transactivator is produced in HeLa cells in amounts sufficiently high for strong activation of transcription though low enough to avoid any detectable squelching effects (31).

The usefulness of heterologous regulatory systems as the one described here depends decisively on quantitative parameters such as the extent of inactivation and the efficiency of activation of gene expression as well as the kinetics of transition from one state to the other. For the *tet* system these parameters were measured in HeLa cell lines that constitutively express tTA and that also contain the luciferase gene stably integrated and under the control of tTA-dependent promoters. The clones characterized thus far express the luciferase gene to various extents. This is not surprising since differences in the integration sites and in the number of integrated transcription units would be expected. However, in all cases the expression of luciferase is sensitive to tetracycline. In some clones tetracycline has the most dramatic effect of reducing the luciferase activity from high levels over several orders of magnitude to background. This demonstrates that in HeLa cells the two promoters *P<sub>hCMV</sub>·1* and *P<sub>hCMV</sub>·2* have no measurable intrinsic activity. Their function strictly depends on tTA. The residual luciferase activity observed in some clones in the presence of tetracycline must therefore be due to position effects.

The tTA-dependent promoters can be kept in a partially activated state by low concentrations of tetracycline. As shown in Fig. 3a, varying the tetracycline concentration between 0 and 0.1 μg/ml allows adjustment of promoter activity within a range of several orders of magnitude. This may allow assessment also of quantitative parameters of gene function *in vivo*.

The activation and inactivation of tTA by the antibiotic appears to be not only an efficient but also a rapid process. When cells from tetracycline-containing medium are shifted to tetracycline-free medium, significant luciferase activity is induced within 4 hr and >20% of the steady-state level is reached within 12 hr after the shift. Interestingly, even the cultures that were only exposed to tetracycline-free medium during the washing procedure before reincubation in tetracycline-containing medium show a small but reproducible increase in luciferase activity that is still detectable after 4 hr (Fig. 3b).

When tetracycline is added to a culture of X1 cells, luciferase activity is reduced ≈10-fold within 8 hr and >50-fold within 12 hr. This decrease is remarkably fast if one takes into account the half-life of luciferase of around 3 hr reported for mammalian cells (measured by cycloheximide inhibition; refs. 33 and 34) and indicates a rapid uptake of tetracycline by HeLa cells followed by a fast and efficient shutdown of transcription. Although the half-life of luciferase and its mRNA remains to be determined in our system, these conclusions are supported by observations in plant cells, where tetracycline inactivates *tetR* within <30 min (23).

Taken together, these data show that tetracycline, unlike IPTG in a eukaryotic *lacR/O*-based system, is able to act fast in cultures of mammalian cells. The possibility of rapidly switching the activity of a tTA-dependent promoter not only is of interest in studying gene function itself but also should allow analysis of mRNA decay rates of individual genes under physiological conditions.

In clone X1 tetracycline reduces luciferase activity reproducibly by five orders of magnitude. This suggests that binding of tetracycline to tTA may lower the association constant between the transactivator and its operator to a much greater extent than that measured for *tetR* (21) and as described for IPTG in the *lacR/O* system, where the binding constant *K<sub>RO</sub>* is reduced only 1000-fold by the inducer (35).

On the other hand, preliminary results obtained in transient experiments with minimal tk promoters fused to single, dimeric, and h ptameric *terO*'s sequences strongly suggest a synergistic effect of multiple tTA binding sites. The efficient inactivation of tTA by tetracycline is therefore most likely due to a large difference in the binding constants of tTA and tTA/tetracycline for the *terO* and the nonlinear effect of tetracycline interfering with a cooperative process.

In conclusion, our data indicate that promoter-activating systems as described here are most promising for regulating individual genes in higher eukaryotic cells for several reasons. (i) For activators, in particular when acting through a cooperative mechanism, intracellular concentrations can be kept low, ensuring an efficient inactivation by the effector—in this case, tetracycline. By contrast, repressors in general compete directly with transcription factors and/or RNA polymerases for binding within a promoter region. In the absence of cooperativity, however, the window at which the repressor concentration is sufficiently high for tight repression but still low enough for efficient induction may be narrow and not easily adjustable in different systems. (ii) In an activating system as described here the synthesis of tTA can be driven by a tissue-specific promoter, whereas the tTA-dependent promoters are expected to function tissue independently, since they may require only general transcription factors in addition to tTA. By contrast, in a repressor-based system in which operators have to be placed within the context of a promoter sequence, an influence on promoter specificity cannot be excluded. (iii) The *ter* system offers specific advantages when compared to the intensely studied *lac* system. For example, *terR* binds tetracycline much tighter ( $k_a \approx 10^9 M^{-1}$ ; ref. 21) than *lacR* complexes IPTG ( $k_a \approx 10^6 M^{-1}$ ; ref. 35). Thus, very low, nontoxic concentrations of tetracycline function effectively. Moreover, a large number of tetracycline derivatives are known, of which some appear to have far superior properties as effectors than the compound used in this study. In this context, it is interesting to note that detailed information on the pharmacological properties of tetracycline, in particular pharmacokinetic parameters, is available, which will facilitate application of this system in transgenic animals.

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